

EVIDENCE FOR THE APPEARANCE OF NOVEL GENE PRODUCTS DURING AMPHIBIAN BLASTULATION*

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Communicated February 20, 1968

Genetic information begins to be transcribed during early cleavage in amphibian embryos,^{1, 2} and the tempo of informational RNA synthesis increases gradually through cleavage and into the early blastula stage. Previous studies from this laboratory^{3, 4} have shown that at this point a remarkable, near embryo-wide acceleration of informational RNA synthesis occurs, resulting during the mid-to-late blastular period in at least a 20-fold increase in the rate of synthesis of heterogeneously sedimenting, DNA-like RNA. Two possibilities present themselves with respect to the significance for the embryo of this first large-scale increase in the level of embryo genome function: either the gene products synthesized in the mid-to-late blastular period are novel, perhaps constituting a precociously transcribed program for gastrular differentiation; or they are at least in part already represented in the enormous stockpiles of informational RNA which our earlier studies have shown to be stored in the embryo from the lampbrush stage of oögenesis.⁵ In the latter case, pregastrular RNA synthesis is to be regarded more as an amplification by the embryo of the patterns of gene activity laid down originally during oögenesis, and indications to this effect now exist in the sea urchin literature. Thus Glisin *et al.*⁶ have shown that virtually all the species of RNA synthesized in the mesenchyme blastula stage are already represented in the informational RNA stored in the unfertilized egg. The generality of homology between the informational RNA stockpile inherited from oögenesis and the informational RNA's synthesized in early embryogenesis is evidently a question of considerable significance in understanding the early function of the embryo genomes, and an object of the present study is to assay this homology in an organism only distantly related to the echinoderms. The experiments which come closest to examining this question in amphibian material are those of Denis,⁷ who demonstrated (in *Xenopus*) that there was no homology between the RNA population in synthesis during mid-to-late gastrulation and the RNA's already present in the embryo between late cleavage and late blastulation. In total informational content the latter RNA population consists in large part of RNA's inherited from oögenesis,⁵ but it is evident that gastrular gene activity cannot be considered *a priori* to be representative of *blastular* gene activity, and that this experiment therefore leaves the novelty of *blastular* transcription unexplored. Previous to the present report, then, the earliest qualitatively new pattern of gene activity in amphibian embryos for which evidence exists⁷ is in the mid-to-late gastrular period, i.e., in a period following rather than preceding the manifest appearance of new, differentiated cell types.

Methods.—Preparation of nucleic acids: Eggs of *Xenopus laevis* were labeled with P³²-phosphate by injection into the gravid female as described by Brown and Littna.¹ P³²-RNA from late blastulae (stage 9 of Nieuwkoop and Faber⁸), unlabeled RNA from early blastulae (stage 7), late blastulae (stage 9), and gastrulae (stage 10), as well as unlabeled

RNA from mature ovarian oöcytes and H³-DNA from *Xenopus* testis, were extracted and extensively purified as described previously.^{5, 9}

Hybridization procedures: RNA-DNA hybridization experiments were carried out with modifications of either the liquid-liquid or the immobilized DNA nitrocellulose filter technique of Gillespie and Spiegelman.¹⁰ Annealing volumes by either method were always 1 ml, and the annealing temperature was 66° for the liquid method and 67° for the immobilized DNA method. After the annealing period, filters were treated with RNase A at 10 µg/ml in 0.05 M Tris-6 × SC (SC is 0.15 M NaCl-0.015 M Na citrate) for 1 hr at room temperature and washed on each side with 50 cc 6 × SC under vacuum, and then by immersion in 50 cc 6 × SC in the cold for 1 hr. They were then hydrolyzed and counted as before.⁹ All annealing solutions were made up in 6 × SC.¹⁰ Hybrids were as stable to 4 hr in RNase as to 1 hr. Saturation annealing times were established anew for each RNA studied.

Competition experiments were always run under conditions of RNA saturation, i.e., with the P³²-labeled reference RNA present in a saturating amount for the 1 µg of DNA used per filter. The rationale for performing hybridization experiments under saturating conditions with respect to the reference RNA has been discussed by us earlier;⁵ briefly, the argument is that in the presence of excess DNA, reference RNA species present in a large number of copies will be hybridized in an amount disproportionately greater than the percentage of the genome complementary to them, as compared to the amount of hybridization occurring with other species of RNA present in fewer copies. Since the amount of reference RNA replaced by competing unlabeled RNA is strictly a function of the ratio of unlabeled to reference RNA, a competition experiment carried out under conditions of excess DNA therefore portrays mainly competition for those genomic sites represented in the reference RNA population in the largest number of copies. While this is perhaps useful in comparing over-all homology between RNA populations, it evidently distorts the competition results in the direction of relative insensitivity to rare species in the reference RNA population, and oversensitivity to frequent species in the reference population, irrespective of the diversity of genomic information (i.e., the proportion of the genome) represented by either. Under the conditions of these experiments at least a large part of the hybridized reference RNA is bound to portions of the genome which are to some extent internally redundant,¹¹ and this is equally true for the other hybridization studies cited.^{6, 7} Our demonstrations of lack of homology between RNA populations are therefore to be interpreted as evidence that *completely different families of genes* are functional in the synthesis of these RNA populations.

Selection of an appropriate hybridization method: Since our object was to test the homology between newly synthesized blastular RNA (stage 9) and other RNA's, an initial problem was to discover the P³²-stage 9 RNA/DNA saturation ratio. Figure 1 presents an experiment determining this point for both the immobilized DNA and liquid-liquid hybridization procedures. The latter is more efficient, as would be expected from its three-dimensional nature, but it is emphasized that the saturation level of hybridization is exactly the same by either method (Fig. 1). This rules out any interference due to DNA-DNA reannealing in the liquid-liquid system, and shows that either technique is applicable. Consequently we have employed both in the following experiments. Note the exceedingly high RNA/DNA ratios needed for saturation, 400:1 and 800:1 for the liquid-liquid and immobilized DNA methods, respectively. These values are to be contrasted to the 1.5 or 2-to-1 ratios needed for saturation with newly synthesized lampbrush chromosome stage oöcyte RNA in the liquid-liquid procedure,^{5, 9} and are due to the comparatively extreme scarcity of the newly synthesized RNA species in the stage 9 embryo RNA preparations. The labeled RNA in the experiment of Figure 1 includes all the RNA synthesized from ovulation to stage 9,¹ but the vast majority of the labeled molecules present are those synthesized in the mid-to-late blastular period (stage 7 to stage 9).³

Figure 2 describes a problem encountered in the use of liquid-liquid hybridization systems operated at these high RNA-to-DNA ratios, and an empirical solution to this prob-

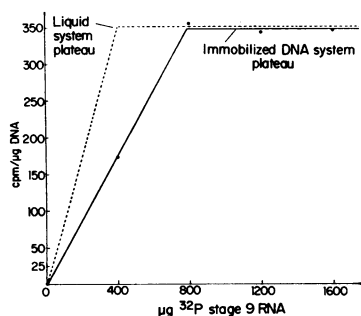


FIG. 1.—Saturation curve obtained by adding increasing amounts of P^{32} -stage 9 RNA to filters containing 1 μ g of immobilized H^3 -DNA, represented by solid points. This curve is superimposed on a saturation curve compiled from liquid-liquid system experiments, and represented by the dotted line.

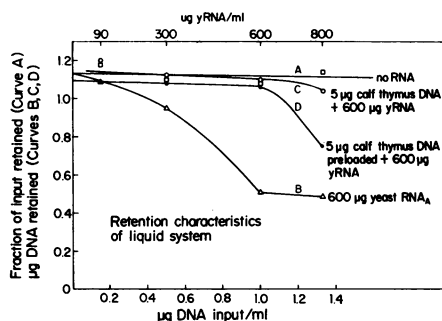


FIG. 2.—Retention of 1 μ g denatured H^3 -DNA in liquid-liquid system. (A) Retention in the absence of added RNA over range 0.1–1.4 μ g/ml input concentration; (B) retention of 1 μ g DNA in the presence of 600 μ g (nuclease-free) yeast RNA, at various concentrations; (C) same, but with 5 μ g denatured calf thymus DNA added as carrier at time of filtration; (D) same, but with 5 μ g denatured calf thymus DNA preloaded on filters.

lem. DNA retention in the filter following the annealing period is a declining, concentration-sensitive function of the amount of RNA present at very high RNA-to-DNA ratios, as can be seen in curve *B* of Figure 2, which shows the effect of added yeast RNA. Without RNA the DNA retention is perfect over a range of input DNA concentrations from 0.1 to 1.4 μ g/ml (curve *A*). By adding heterologous carrier DNA to the solution at the end of the annealing period, however (*C*), or even by preloading it on the filters (*D*), retention of the hybridizing H^3 -DNA can be rendered perfect. Under these conditions no RNase-resistant binding of RNA by the carrier DNA occurs. In the liquid-liquid system experiments which follow, 10 μ g calf-thymus DNA is added per sample.

In order to obtain proper competition curves it is imperative to operate at saturating times as well as concentrations. The time required to approach saturation is variable, depending on the saturation ratios and the concentrations of both RNA and DNA. Figure 3 shows two time curves for the liquid-liquid system in which the same amount of RNA (400 μ g) is exposed to either 1 μ g of DNA (saturating ratio) or 2 μ g of DNA. The true saturation plateau of the 1- μ g curve requires about 2.5 times as long to attain as the subsaturation, or "RNA exhaustion," curve at 2 μ g of DNA. If the specific activities of the various RNA species are randomly distributed as one proceeds up the ordinate in Figure 3, the number of RNA molecules hybridized to each DNA increases by the same factor, 2.5, if the saturation and subsaturation curves are compared. All liquid-liquid experiments were carried out with 30 hr annealing time. The hybrids remain stable for 72 hr at 66°C, and absolutely no further increase in hybridization is obtained. Hybrids in the immobilized DNA system remain stable for longer than this and likewise no further hybridization occurs beyond the initial time-saturation point.

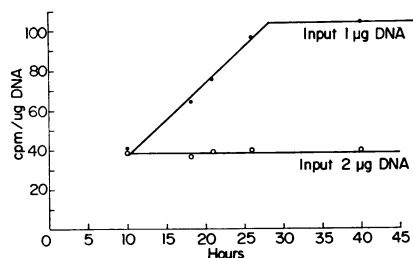


FIG. 3.—Hybridization after various annealing times between 400 μ g of P^{32} -stage 9 RNA and 1 or 2 μ g H^3 -DNA in the liquid-liquid system.

The test of the liquid-liquid hybridization

competition system is shown in Figure 4, which presents a dilution curve obtained by adding increasing amounts of unlabeled stage 9 RNA to an annealing mixture of P^{32} -stage 9 RNA and 1 μ g of H^3 -DNA. In Figure 4A the data are plotted directly and in Figure 4B a comparison between the theoretical and experimental dilution curves is presented. The decrease in the hybridization of the reference P^{32} -RNA is a linear function of the proportion of reference RNA in the mixture, as theoretically predicted, and the slope is essentially the theoretical one. Also demonstrated in Figure 4A is the fact that absolutely no competition is registered with a heterologous RNA preparation (yeast) and that the quantities of competing RNA lie within the range of the normal saturation level with increasing amounts of reference RNA.

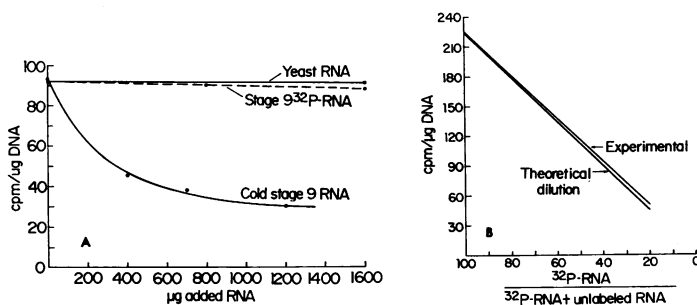


FIG. 4.—Competition characteristics of liquid-liquid system.

(A) Competition between increasing amounts of unlabeled stage 9 RNA and 400 μ g P^{32} -stage 9 RNA for binding sites in 1 μ g H^3 -DNA. Lack of interference with plateau level hybridization of the P^{32} -RNA by excess P^{32} -RNA or yeast RNA is also shown.

(B) Pooled results of two competition curves obtained with different RNA preparations, normalized to the same starting specific activity, and compared to the theoretical dilution curve.

Results.—*Tests for homology between newly synthesized blastular RNA and the RNA stored in the mature oöcyte:* A direct assay of possible homology was made by challenging a saturating annealing mixture of 400 μ g stage 9 P^{32} -RNA and 1 μ g *Xenopus* H^3 -DNA with unlabeled RNA from mature ovarian oöcytes, using the quantitative liquid-liquid competition system described in Figures 1–4 above. The result of this experiment is shown in Figure 5. Absolutely no homology is apparent. Thus the ovarian oöcyte RNA, despite its very large informational content,⁹ displays no more homology for those informational RNA's which are in synthesis up to the late blastula stage than does the yeast RNA of Figure 4A.

A possible loophole exists in this interpretation, however. It is conceivable that homology does exist, but only between species of RNA which are present in the mature oöcyte preparation in a very small number of copies compared to their number in the P^{32} -labeled reference RNA population. If the disparity between these copy numbers were sufficiently great, the ratio unlabeled/labeled molecules might never be great enough to result in a significant, observable competition. To eliminate this possibility, a two-step saturation experiment was performed in which an aliquot of *Xenopus* DNA was first saturated with mature oöcyte RNA, and then exposed to saturating amounts of P^{32} -stage 9 RNA. Given the existence of a true initial saturation with the oöcyte RNA, then

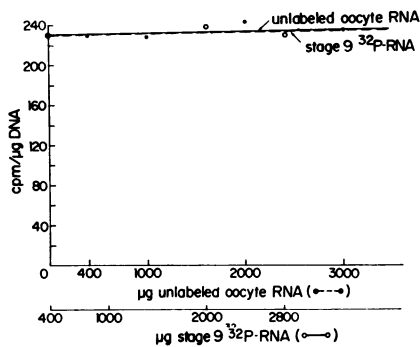


FIG. 5.—Test for homology between mature oocyte RNA and newly synthesized stage 9 RNA, using the liquid-liquid system. Lack of interference by excess P^{32} -stage 9 RNA is also shown. Each sample contained $1\ \mu\text{g}$ H^3 -DNA, $400\ \mu\text{g}$ stage 9 P^{32} -RNA, and the amounts of added RNA shown on the abscissa.

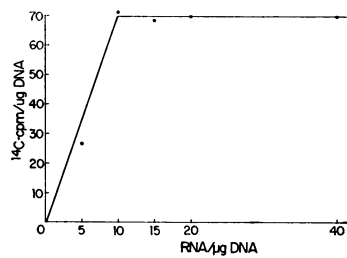


FIG. 6.—Saturation curve obtained with increasing ratios of C^{14} -methyl-labeled mature oocyte RNA to H^3 -DNA. Each sample contained $5\ \mu\text{g}$ of DNA immobilized on a filter. The RNA was methylated as described by Smith *et al.*¹³ $10.5\ \text{mg}$ RNA were exposed to $32\ \mu\text{g}$ C^{14} -dimethyl sulfate in $2.6\ \text{ml}$ $0.1\ M$ phosphate buffer pH 7.5 at room temperature for 4 hr. The RNA was then twice precipitated in ethanol, purified of adherent counts by passage through a G-25 Sephadex column, and reprecipitated.

irrespective of the relative copy number, homology between the oocyte RNA's and the newly synthesized blastular RNA's should be observed as a decrease from the usual saturation level of hybridization when the blastular P^{32} -RNA and the DNA bearing the prehybridized oocyte RNA are allowed to hybridize. To carry out this experiment it is necessary to use the immobilized DNA method, so that the DNA can be transferred from the first to the second RNA solution merely by transferring the filter. Furthermore, it is necessary to label the oocyte RNA preparation so that the saturating oocyte RNA/DNA ratios can be ascertained, and so that the retention in the second step of RNA molecules hybridized during the first step can be monitored. In *Xenopus* mature oocyte RNA cannot be labeled metabolically,^{9, 12} and advantage was taken of a recent report by Smith *et al.*,¹³ who have shown that RNA can be methylated *in vitro* with C^{14} -dimethyl sulfate without affecting its behavior in hybridization systems. In Figure 6 a saturation curve obtained with C^{14} -methylated ovarian oocyte RNA is presented (see legend to Fig. 6 for details of the methylation procedure, which is essentially that of Smith *et al.*¹³). Mainly the guanine residues are methylated, and though in our hands the extent of guanine methylation varies somewhat depending on the type of RNA used, all RNA's are probably methylated. Note in Figure 6 the relatively low saturating RNA/DNA ratios (10:1): this is what is to be expected on the basis of the low ratios required with metabolically labeled lampbrush stage RNA,^{5, 9} since the mature oocyte contains a massive informational RNA stockpile inherited directly from the lampbrush stage of oögenesis.⁹ In this comparison allowance must also be made for the two- to threefold lower efficiency of the immobilized DNA method.

In Table 1 the results of the two-step saturation experiment are presented. The procedure used was to incubate filters containing $1\ \mu\text{g}$ H^3 -DNA with the RNA solutions listed in the table for 24 hours, and then remove the filters and

TABLE 1. *Two-step test for homology between newly synthesized blastular RNA's and mature oocyte RNA.**

Sample	Anneal first with	Anneal second with	C ¹⁴ cpm/ μg DNA	P ³² cpm/ μg DNA
A	10 μg C ¹⁴ -oocyte RNA	(filter not changed)	50.4	—
B	10 μg C ¹⁴ -oocyte RNA	6 × SC	47.4	—
C	6 × SC	400 μg stage 9 P ³² -RNA	—	266.6
D	10 μg cold oocyte RNA	10 μg C ¹⁴ -oocyte RNA	0	—
E	400 μg cold stage 9 RNA	400 μg P ³² -stage 9 RNA	—	0
F	10 μg C ¹⁴ -oocyte RNA	400 μg P ³² -stage 9 RNA	47.1	257.6
G	40 μg C ¹⁴ -oocyte RNA	400 μg P ³² -stage 9 RNA	52.0	248.9
H	120 μg C ¹⁴ -oocyte RNA	400 μg P ³² -stage 9 RNA	51.1	257.5

* DNA retention in all samples was in the range 0.91–1.04 of the 1-μg input. The first incubation was for 24 hr, the second for 30 hr. After the second incubation the filters were processed as described in *Methods*, and counted in a scintillation spectrometer using a three-channel mode permitting simultaneous assay of H³, C¹⁴, and P³². All samples were in duplicate except for C and G, which were in triplicate, and H, which was single.

wash them thoroughly by sucking 50 cc of 6 × SC through them under vacuum from each side in turn. The filters were next transferred to a different vial containing the second RNA solution. As the table shows, the system works perfectly, and we encountered none of the difficulties experienced by Birnboim *et al.*¹⁴ in a similar experiment, on the basis of which these authors question the general competition hybridization technique as applied to animal cell RNA's. Although it is obvious that this technique must be applied carefully, the high degree of specificity of the hybrids, their stability, and the near-theoretical operation of the competition procedure (Fig. 4, Table 1) make RNA-DNA hybridization a reliable and uniquely valuable research tool, providing that the appropriate controls and safeguards are observed.

The first two samples of Table 1, A and B, establish the saturation plateau value for C¹⁴-methyl-labeled mature ovarian oocyte RNA, and show that exposure to 67° 6 × SC alone for a subsequent 30 hours does not result in leaching out of hybridized counts. The third example, C, establishes the saturation plateau level for stage 9 P³²-RNA. In D and E it is demonstrated that if saturating amounts of unlabeled oocyte RNA and unlabeled stages 9 blastular RNA, respectively, are hybridized in the first step, the homologous labeled RNA's are completely blocked from hybridization in the second step. This result shows that the two-step saturation procedure responds completely to complete homology in the tested RNA's, so that initial saturation of the set of homologous DNA sites renders these sites totally inaccessible to the same RNA thereafter. Samples F, G, and H show that *presaturation with C¹⁴-methyl-labeled oocyte RNA does not significantly affect the level of hybridization of newly synthesized blastular RNA*, and the C¹⁴ counts show that there has been complete retention of hybridized oocyte RNA during the second step. Retention of the initially hybridized species of RNA in our experience is absolute irrespective of whether a totally homologous or a heterologous RNA is added in the second step. Though exposure to supersaturation quantities of oocyte RNA in the first step of the procedure should theoretically not affect the result, as an added precaution 4 and 12 times the saturating amounts of oocyte RNA were applied in samples G and H. No further effect on the stage 9 hybridization level was observed in these

samples. Relative differences in RNA copy number would thus appear to be ruled out as an alternative interpretation for the experiment of Figure 5. It is concluded that there is in fact no significant homology between the RNA's synthesized by stage 9 of embryogenesis and the RNA's stored in the mature oöcyte. Therefore the spectrum of genes functioning as early as stage 9 is a completely novel one.

On the significance of mid-to-late blastular gene transcription: As pointed out above, almost all the counts incorporated in stage 9 late blastular RNA represent synthesis during the mid-to-late blastular period.³ Nevertheless, informational RNA synthesis does occur earlier,¹⁻³ and it is of interest to ascertain whether the spectrum of gene products represented in stage 9 RNA is also novel with respect to the RNA's present in the stage 7 (early blastula) embryo. Comparison for homology between newly synthesized stage 9 RNA's and the RNA's present in stage 10 gastrulae is also of interest, in that such a comparison would indicate whether the blastular gene products are confined to the stage of their synthesis or are retained beyond the end of blastulation into gastrulation. In Figure 7

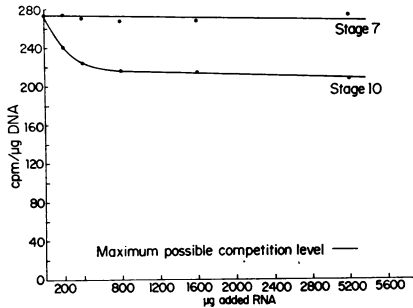


FIG. 7.—Test for homology in liquid-liquid system between P^{32} -stage 9 RNA and unlabeled RNA from stage 7 (early blastula) and stage 10 (early gastrula). Each sample contained 400 μ g stage 9 P^{32} -RNA, 1 μ g H^3 -DNA, and the amounts of unlabeled RNA shown on the abscissa. The maximum possible competition level at 3200 μ g added RNA is taken from the dilution curves of Fig. 4; the competition registered by stage 10 RNA is 28% of this maximum.

both of these experiments are presented. It is clear that no significant homology is evident between the RNA's being synthesized in stages 8 and 9 and the RNA's present in the stage 7 embryo, so that the mid-to-late blastular gene products can be regarded as absent prior to mid-blastulation. This means that the massive acceleration of gene activity which occurs at this point represents the activity of qualitatively new genes. About 28 per cent of the hybridized RNA counts are competed for by the gastrular RNA's, on the other hand, and thus either a significant fraction of the blastular gene products remains present in the embryo or the genes (or at least the *families* of genes¹¹) on which they were synthesized remain active in the stage 10 embryo, or both. The actual proportion of the stage 9 gene products homologous with stage 10 gene products cannot be stated since it is not known whether the average specific activity of that portion of the stage 9 P^{32} -RNA population which is homologous with stage 10 RNA is the same as that of the nonhomologous stage 9 RNA. The latter, however, is apparently the larger fraction, and we conclude that the majority of the genes active in blastulation probably synthesize RNA's destined for immediate use.

Conclusions.—These experiments present a picture of very rapidly changing patterns of gene activity early in amphibian development. Thus, in contrast to

the case found in the sea urchin,⁶ the gene products synthesized in amphibian blastulation are the products of different genes than those functional during oögenesis in preparing the inherited informational RNA stockpile of the embryo, and in fact are distinct from all gene products present as late as the onset of blastulation. Within a few hours after this, a massive acceleration of gene activity occurs, in the mid-to-late blastular period, and a range of novel gene products is produced. Most of these new informational RNA's appear to be utilized immediately, since they have disappeared by the next stage, the early gastrula. A significant proportion of the blastular RNA's is, however, retained into gastrulation, or the genes synthesizing them remain active in the gastrula, or both. In any case, a portion of the spectrum of informational gene products characterizing the definitive gastrula is established in blastulation, and in this sense our evidence is a biochemical confirmation of the supposition that transcription for gastrular differentiation begins well in advance of gastrular morphogenesis.

The question arises as to whether gene products formed prior to the mid-to-late blastular period in the amphibian embryo might be homologous with maternal informational RNA, as is sea urchin hatching blastular RNA.⁶ It is difficult to say whether there is a corresponding stage in amphibian development, and, if so, whether it is earlier than stage 7. RNA's formed previous to the stage 7-9 period were not investigated in the present study because their rate of synthesis is so low as to preclude adequate labeling with the methods used. This is nevertheless an important problem, since control of the initial patterns of gene activity in the embryo is likely to depend on maternal instructions.

It is emphasized that the results presented in this report establish the existence of stage-specific patterns of gene activity at the earliest stages of development so far known in amphibian embryogenesis. The rapid change in these patterns of gene activity and the immediate utilization of short-lived gene products prior to the first large-scale differentiation of tissue layers serve as a direct index of early control over morphogenetic events on the part of the embryo genome.

* This work was supported by American Cancer Society grant E-334 B and by National Institute of Child Health and Development grant HD 02412-02.

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